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A Novel *cis*-Acting Element in an Arabidopsis Gene Is Involved in Responsiveness to Drought, Low-Temperature, or High-Salt Stress

Kazuko Yamaguchi-Shinozaki[a,b] and Kazuo Shinozaki [a,1]

[a] Laboratory of Plant Molecular Biology, The Institute of Physical and Chemical Research (RIKEN), Tsukuba Life Science Center, 3-1-1 Koyadai, Tsukuba, Ibaraki 305, Japan

[b] Biological Resources Division, Japan International Research Center for Agricultural Sciences (JIRCAS), Ministry of Agriculture, Forestry and Fisheries, 1-2 Ohwashi, Tsukuba, Ibaraki 305, Japan

Two genes, *rd29A* and *rd29B*, which are closely located on the Arabidopsis genome, are differentially induced under conditions of dehydration, low temperature, high salt, or treatment with exogenous abscisic acid (ABA). It appears that *rd29A* has at least two *cis*-acting elements, one involved in the ABA-associated response to dehydration and the other induced by changes in osmotic potential, and that *rd29B* contains at least one *cis*-acting element that is involved in ABA-responsive, slow induction. We analyzed the *rd29A* promoter in both transgenic Arabidopsis and tobacco and identified a novel *cis*-acting, dehydration-responsive element (DRE) containing 9 bp, TACCGACAT, that is involved in the first rapid response of *rd29A* to conditions of dehydration or high salt. DRE is also involved in the induction by low temperature but does not function in the ABA-responsive, slow expression of *rd29A*. Nuclear proteins that specifically bind to DRE were detected in Arabidopsis plants under either high-salt or normal conditions. Different *cis*-acting elements seem to function in the two-step induction of *rd29A* and in the slow induction of *rd29B* under conditions of dehydration, high salt, or low temperature.

INTRODUCTION

Plants respond to conditions of severe environmental changes or stresses, such as drought, low temperature, or high salt, with a number of physiological and developmental changes. Abscisic acid (ABA) appears to play an important role in the ability of plants to tolerate these stresses (Mansfield, 1987). ABA is produced under such conditions and plays important roles in tolerance against these stresses (Mansfield, 1987). Drought or high-salt conditions induce dehydration of plant cells, which may trigger physiological and biochemical responses against such stresses.

Recently, a number of genes have been described that respond to dehydration and low temperature at the transcriptional level (see reviews by Skriver and Mundy, 1990; Thomashow, 1990; Bray, 1991). Most of the genes that have been studied to date are also induced by ABA (Mundy and Chua, 1988; Close et al., 1989; Bartels et al., 1990; Hajela et al., 1990; Kurkela and Franck, 1990; Pla et al., 1991; Lång and Palva, 1992). It appears that dehydration or low temperature triggers the production of ABA, which, in turn, induces various genes. Many genes that respond to ABA are also expressed at the late stages of embryogenesis during the development of seeds (Skriver and Mundy, 1990) and are thought to function in the protection of cell dehydration (Dure et al., 1989; Skriver and Mundy, 1990). *cis*- and *trans*-acting factors involved in ABA-induced gene expression have been analyzed extensively. A conserved sequence, PyACGTGGC, has been reported to function as an ABA-responsive element (ABRE) in many ABA-responsive genes (Marcotte et al., 1989; Mundy et al., 1990; Bray, 1991). cDNAs encoding DNA binding proteins that specifically bind to the ABRE have been cloned and shown to contain the basic domain/leucine zipper (bZIP) structure (Guiltinan et al., 1990; Oeda et al., 1991). Recently, different *cis*-acting elements have been reported to function in ABA-induced gene expression during seed maturation of maize (Hattori et al., 1992) and in transgenic tobacco (Lam and Chua, 1991). Several ABA-inducible genes require protein biosynthesis for their induction, whereas most do not require protein synthesis (Peña-Corés et al., 1989; Yamaguchi-Shinozaki and Shinozaki, 1993b).

Several reports have described genes that are induced by dehydration but are not responsive to exogenous ABA treatment (Guerrero et al., 1990; Yamaguchi-Shinozaki et al., 1992). Low-temperature–inducible genes have been shown to be regulated by three separate signal pathways, one of which is ABA independent (Gilmour and Thomashow, 1991; Nordin et

[1] To whom correspondence should be addressed.

al., 1991). These findings suggest the existence of ABA-independent as well as ABA-dependent signal transduction cascades between the initial signal of drought or cold stress and the expression of specific genes. Although the *cis*- and *trans*-acting factors of ABA-responsive genes have been analyzed extensively, our understanding of the molecular mechanism of ABA-independent gene expression by drought or low temperature, and their signal pathways, is still limited. Therefore, it is important to investigate the *cis*-acting elements that function in ABA-independent gene expression by dehydration, high salt, or cold stress.

We have isolated at least four dehydration-responsive genes that show ABA-independent expression in Arabidopsis (Yamaguchi-Shinozaki et al., 1992; Yamaguchi-Shinozaki and Shinozaki, 1993a). The transcription of genes that hybridize to RD29 cDNA is induced very rapidly and at a high rate 20 min after the start of dehydration, and this transcription is followed by a second induction phase that begins after ~3 hr of dehydration (Yamaguchi-Shinozaki et al., 1992). The levels of RD29 mRNA change differently in response to dehydration, low temperature, salt stress, or exposure to ABA (Yamaguchi-Shinozaki and Shinozaki, 1993a, 1993c). Two genes corresponding to RD29, *rd29A* and *rd29B*, are located in tandem in an 8-kb region of the Arabidopsis genome and encode hydrophilic proteins (Yamaguchi-Shinozaki and Shinozaki, 1993a). Dehydration induces *rd29A* mRNA with a two-step kinetics, whereas *rd29B* is induced within 3 hr of dehydration. The expression of both genes, however, is stimulated ~3 hr after treatment with ABA. In the dehydration conditions of the present study, endogenous ABA began to accumulate 2 hr after dehydration started and reached its maximum at 10 hr (Kiyosue et al., 1994), which suggests that the first rapid induction of *rd29A* is not mediated by endogenous ABA. The ABA-insensitive (*abi1*) mutation decreased the level of induction of the *rd29A* mRNA by dehydration at 10 hr. Therefore, it appears that *rd29A* has at least two *cis*-acting elements. One seems to be involved in an ABA-associated slow response to dehydration, and the other may function in ABA-independent rapid induction. Nordin et al. (1993) have isolated low-temperature–inducible (*lti*) genes of Arabidopsis using differential hybridization. Two of the *lti* genes, *lti78* and *lti65*, are identical with dehydration-inducible *rd29A* and *rd29B*, respectively. They also reported differential expression of these genes by low temperature, exogenous ABA, or drought. Expression of *lti78* is induced mainly by low temperature, whereas the expression of *lti65* is induced by both ABA and drought. Nordin et al. also realized that the induction of *lti78* or *rd29A* follows separate signal pathways under conditions of low temperature, drought, or ABA treatment (Nordin et al., 1991, 1993). The drought induction of *lti65* or *rd29B* is ABA dependent.

To analyze the *cis*-acting elements involved in the ABA-independent gene expression of *rd29A*, we constructed fusion genes with the *rd29A* promoter fused to the β-glucuronidase (*GUS*) reporter gene and transformed Arabidopsis and tobacco plants with these constructs. The *GUS* reporter gene driven by the *rd29A* promoter was induced at significant levels in transgenic Arabidopsis by conditions of dehydration, low temperature, or high salt or by treatment with ABA (Yamaguchi-Shinozaki and Shinozaki, 1993a). In the present study, we investigated the *cis*-acting elements involved in dehydration-responsive expression in the *rd29A* promoter and identified a novel *cis*-acting element involved in the first rapid response of *rd29A* to dehydration or high salt. This element seems to function in the induction of *rd29A* by low temperature but not in its ABA-responsive expression. In the present report, we also discuss the *cis*-acting elements involved in the second slow expression of *rd29A* and the ABA-responsive expression of *rd29B*. Different signal transduction pathways seem to exist between the initial signals of dehydration, high salt, or low temperature and the expression of the two *rd29* genes.

RESULTS

Differential Induction of Two *rd29* Genes by Dehydration, Low-Temperature, High-Salt, or ABA Treatment

RNA gel blot analysis was performed with DNA fragments corresponding to the 3′ flanking regions of the *rd29* genes to analyze specific expression of these genes. The *rd29A* gene was induced within 20 min after dehydration began and was strongly expressed after 2 hr (Yamaguchi-Shinozaki and Shinozaki, 1993a), as shown in Figure 1. By contrast, the *rd29B* mRNA did not accumulate to a detectable level until 2 hr after dehydration. The maximum level of *rd29B* mRNA detected at 10 hr was approximately one-tenth of the level of *rd29A* mRNA at 2 hr.

The *rd29A* mRNA was induced within 5 hr after exposure to low temperature (4°C) and was detectable for at least 24 hr (Figure 1). However, *rd29B* mRNA did not accumulate within 24 hr. The *rd29A* mRNA was detected within 1 hr after the initiation of high-salt treatment, as was the case for dehydration, and reached its maximum at 5 hr (Figure 1). By contrast, *rd29B* was induced slowly at ~2 hr after high-salt treatment. When, as a control, the plants were transferred from agar to water, rapid but weak expression of the *rd29A* mRNA was detected (Figure 1). Thus, *rd29A* mRNA appeared to be induced by the transfer of plants from growth conditions of low osmotic potential to those of high osmotic potential and vice versa. Heat stress had no effect on the induction of either *rd29* gene (K. Yamaguchi-Shinozaki and K. Shinozaki, unpublished observation).

Deletion Analysis of the Promoter Regions of *rd29A* or *rd29B* Involved in the Dehydration-Responsive Expression

RNA gel blot analysis using gene-specific probes (Yamaguchi-Shinozaki and Shinozaki, 1993a) indicated that the *rd29A* promoter contained at least two independent *cis*-acting elements

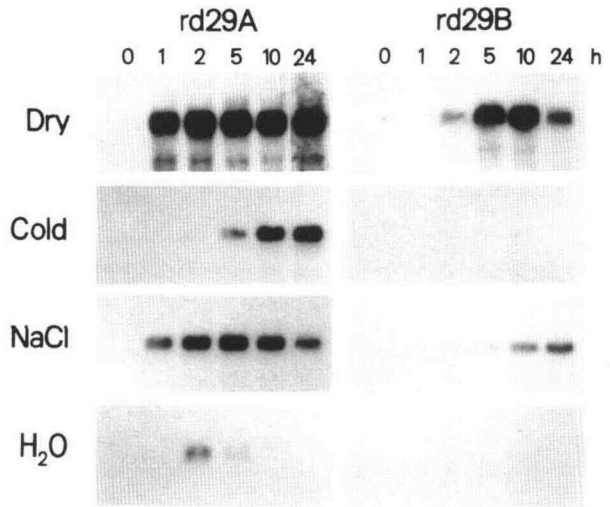

Figure 1. Expression of *rd29A* and *rd29B* in Response to Dehydration, Low-Temperature, or High-Salt Stresses.

Each lane was loaded with 20 μg of total RNA from 3- to 4-week-old unbolted Arabidopsis plants that had been dehydrated (Dry), transferred to and grown at 4°C (Cold), transferred from agar plates for hydroponic growth in 250 mM NaCl (NaCl), or transferred from agar plates to water (H_2O) for hydroponic growth, as described in Methods. The number above each lane indicates the number of hours after the initiation of treatment prior to isolation of RNA. RNA was analyzed by RNA gel blotting with gene-specific probes from the 3′ flanking sequences of *rd29A* and *rd29B*, as described previously by Yamaguchi-Shinozaki and Shinozaki (1993a).

that were involved in ABA-responsive or ABA-independent induction by water deficiency. By contrast, the *rd29B* promoter seems to contain at least one *cis*-acting element involved in ABA-responsive gene expression. To examine this hypothesis, we constructed a chimeric gene, as shown in Figure 2, that consisted of the deleted series of promoter regions of the two *rd29* genes fused to the *GUS* reporter gene, and the fused genes were transferred into Agrobacterium for transformation of tobacco and Arabidopsis plants. Eight gene fusions (*rd29A–GUS*) with the *rd29A* promoter 5′ deleted to −861, −694, −417, −323, −268, −111, −74, and −61 were used for the analysis of dehydration-responsive expression of the *GUS* reporter gene (Figure 2A).

We analyzed 15 independent transgenic tobacco plants for expression of each *rd29A–GUS* fusion gene with the deleted promoter. The −861 transformant exhibited dehydration-induced expression of GUS activity that was 16 times higher than low basal levels (Figure 2A). The −694, −417, −323, and −268 transformants showed similar levels of induction (14.1- to 23.5-fold increase). However, the −111, −74, and −61 transformants exhibited little dehydration-induced expression of GUS activity (1.7- to 2.2-fold increase). These observations indicate that the 157-bp promoter region between −268 and −111 includes *cis*-acting elements involved in the dehydration-induced expression of *rd29A*. The level of the GUS activity of the −323 dehydrated transformant decreased compared with that of the −417 transformant, which suggests an enhancer-like element between −417 and −323. Similar results were obtained with transgenic Arabidopsis using RNA gel blot analysis to study the induction of the *GUS* mRNA driven by deleted *rd29A* promoters (K. Yamaguchi-Shinozaki and K. Shinozaki, unpublished observations).

Five fusion genes (*rd29B–GUS*) with the *rd29B* promoter 5′ deleted to −946, −464, −333, −169, and −51 were used to analyze the induction of the *GUS* gene under drought

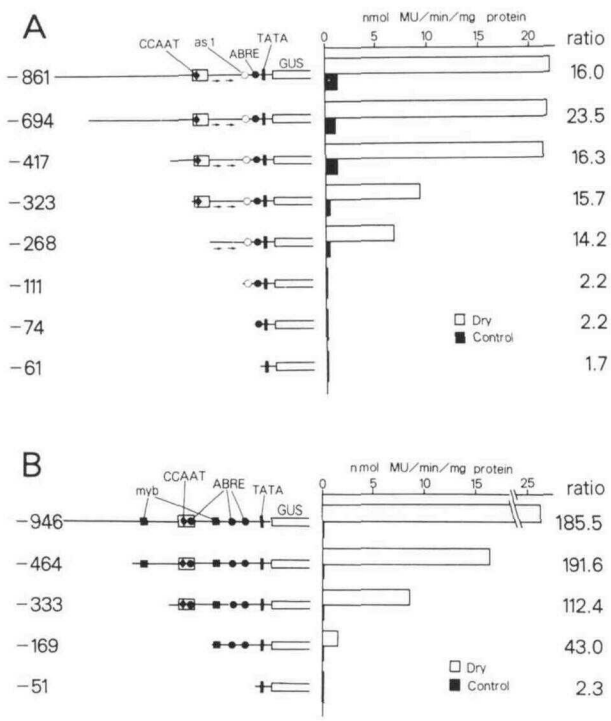

Figure 2. 5′ Deletion Analysis of the *rd29A* and *rd29B* Promoters for the Dehydration-Responsive Induction of the *GUS* Reporter Gene in Transgenic Tobacco.

(A) Deletion fragments of the *rd29A* promoter fused to *GUS*.
(B) Deletion fragments of the *rd29B* promoter fused to *GUS*.
The 5′ terminal deletion fragments of the *rd29A* or *rd29B* promoter fused to the *GUS* reporter gene were introduced into the tobacco chromosome via Agrobacterium-mediated transformation. Leaves of transformed tobacco plants were dehydrated on filter papers in low-light conditions, as described in Methods. Schematics of the 5′ terminal deletions of the promoters fused to the *GUS* reporter gene are shown on the left. Average GUS activities in tobacco leaves before (filled boxes) and after (open boxes) the dehydration treatment were measured and are shown on the right. The multiplicities of the induction of GUS activity by dehydration are shown on the right (ratio). GUS activity was measured in 15 independently obtained transgenic plants for each construct. DNA sequences that are similar to the reported *cis*-acting element are shown as follows: CCAAT, closed diamond; as1, open circles; ABRE, closed circles; TATA, closed rectangles; myb, filled boxes. Arrows indicate 20-bp direct sequences.

conditions in transgenic tobacco (Figure 2B). Under these conditions, the −946, −464, and −333 transformants increased the induction of GUS activity 112 to 192 times higher than basal levels. Dehydration-induced *GUS* expression was still observed with the −169 transformant (43-fold increase), but the −51 transformant showed almost no induction (2.3-fold increase). These results indicated that the 118-bp region between −169 and −51 may contain *cis*-acting elements that are involved in the dehydration-responsive expression of *rd29B*. As the levels of GUS induction were gradually reduced from −946 to −169, there appeared to be several enhancer-like elements between −946 and −169. Similar results were obtained with transgenic Arabidopsis using RNA gel blot analysis of the *GUS* mRNA (K. Yamaguchi-Shinozaki and K. Shinozaki, unpublished observation).

We compared the promoter regions between *rd29A* and *rd29B* and found several conserved sequences and several *cis*-acting elements, as shown in Figure 3. Highly conserved regions were found around the TATA box sequences of both promoters. Two ABRE-like sequences and a myb recognition sequence (PyAACT/GG) were found between −169 and −51 in the *rd29B* promoter, while the corresponding region of *rd29A* showed only one ABRE-like and one activator sequence (as1)-like sequence, which functions in root-specific expression. ABRE has been found in the promoter regions of many ABA-inducible genes (Marcotte et al., 1989; Yamaguchi-Shinozaki et al., 1989; Skriver and Mundy, 1990). The 118-bp region between −169 and −51 was necessary for the dehydration-responsive expression of *rd29B* (the −169 transformant), but its corresponding region in *rd29A* (the −111 transformant; Figure 3) did not function under drought conditions. We found 20-bp direct repeat sequences in the 162-bp region between −268 and −111 of the *rd29A* promoter region, which is necessary for its dehydration-induced expression, but we did not find this 20-bp sequence in the *rd29B* promoter. A 39-bp conserved sequence containing a CCAAT sequence and GC-rich regions was found in both promoters (Figures 2 and 3). We found typical ABRE in the 39-bp region of the *rd29B* promoter but not in the corresponding region of the *rd29A* promoter. In the upstream regions of *rd29B*, we found a myb recognition sequence.

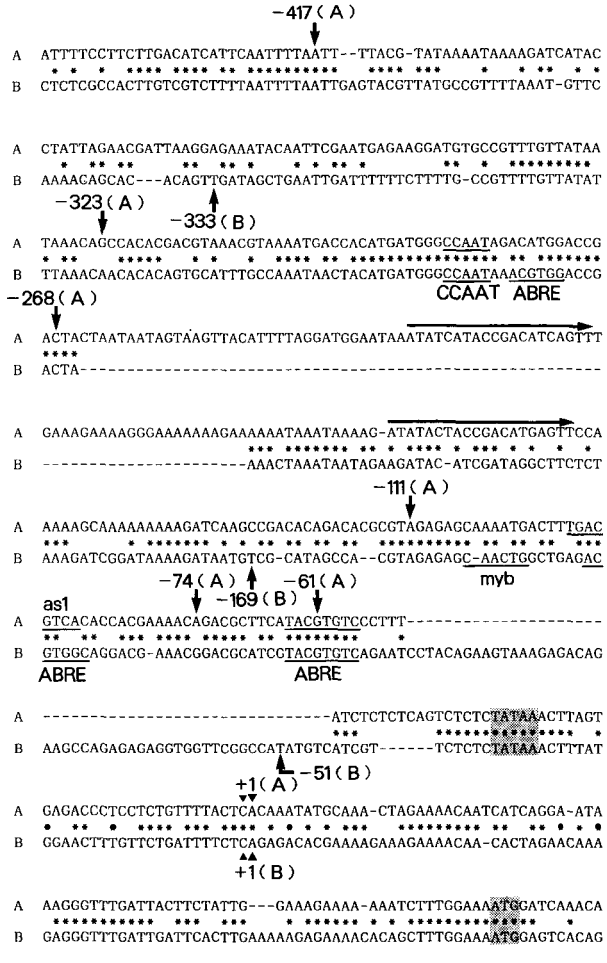

Figure 3. Alignment of the Promoter Regions of *rd29A* and *rd29B*.

The 549-bp 5′ flanking region of *rd29A* was aligned with the 504-bp 5′ flanking region of *rd29B*. Asterisks indicate identical nucleotides in the two promoter regions. Transcriptional initiation sites that were determined by primer extension are marked by arrowheads. The initiation codons (ATG) and the TATA boxes are shaded. The 39-bp conserved regions in the two promoters are shaded. The 20-bp direct repeat sequences in the *rd29A* promoter are indicated with arrows. CCAAT box, ABRE, myb recognition sequence, and *as1* are underlined. Numbers indicate the nucleotide positions used for the 5′ deletion analysis of *rd29A* and *rd29B* promoters in transgenic plants, as shown in Figure 2.

Differential Tissue-Specific Expression of *rd29A-GUS* and *rd29B-GUS* in Dehydrated Transgenic Arabidopsis

We analyzed the distribution of the expression of the *rd29A–GUS* and *rd29B–GUS* fusion genes in transgenic Arabidopsis plants. The *rd29A* and the *rd29B* promoters used for the constructions included the sequence between −861 and +97 and that between −946 and +99, respectively. Figures 4A and 4D show the results of expression of the −861 *rd29A–GUS* fusion gene in transgenic Arabidopsis plants raised under water-deficit and normal conditions, respectively. Figures 4B and 4E show the expression pattern of the −946 *rd29B–GUS* fusion gene in transgenic Arabidopsis plants raised under similar conditions. Weak expression of the −861 *rd29A–GUS* and the −946 *rd29B–GUS* fusion genes was observed in several regions of rosette plants raised under normal growth conditions (Figures 4D and 4E, respectively). The dehydration of transgenic Arabidopsis plants containing the −861 *rd29A–GUS* fusion gene for 8 hr under the same conditions as those used for RNA gel blot analysis strongly induced *GUS* expression in all organs and tissues (Figure 4A). By contrast, *GUS* was strongly expressed in leaf and petiole tissues but not in root tissues of transgenic Arabidopsis plants that contained the −946 *rd29B–GUS* construct (Figure 4B). These

observations indicate that both *rd29A* and *rd29B* promoters contain *cis*-acting elements that respond to dehydration. Further, these observations indicate that the *rd29A* promoter functions in all vegetative tissues of Arabidopsis plants, while the *rd29B* promoter functions only in aerobic tissues during dehydration.

We found an as1-like sequence in the *rd29A* promoter but not in the *rd29B* promoter. Previous studies have reported that as1 functions as a *cis*-acting element involved in root-specific expression in the cauliflower mosaic virus (CaMV) 35S promoter (Benfey et al., 1989). We observed expression of *GUS* in the root tissues of dehydrated transgenic plants that contained the −268 *rd29A–GUS* fusion gene having an as1-like sequence (Figure 4C). By contrast, no induction of GUS activity was observed in tissues that contained a 162-bp fragment between −274 and −113 that had been fused to the −61 *rd29A–GUS* construct, as shown in Figure 5, and that did not contain the as1-like sequence (Figure 4F). These observations indicate that as1 functions as a *cis*-acting element involved in root-specific expression.

Promoter Region with the 20-bp Direct Repeat Is Required for Dehydration-Responsive Expression of *rd29A*

We analyzed the *cis*-acting elements involved in the dehydration-responsive expression of *rd29A* only, because *rd29A* seems to be controlled by two independent signal pathways. A "loss-of-function" experiment demonstrated that the 162-bp region between −268 and −111 contained *cis*-acting elements involved in dehydration-responsive expression (Figure 2A). A "gain-of-function" experiment was then performed and demonstrated that the 162-bp region contains positive regulatory

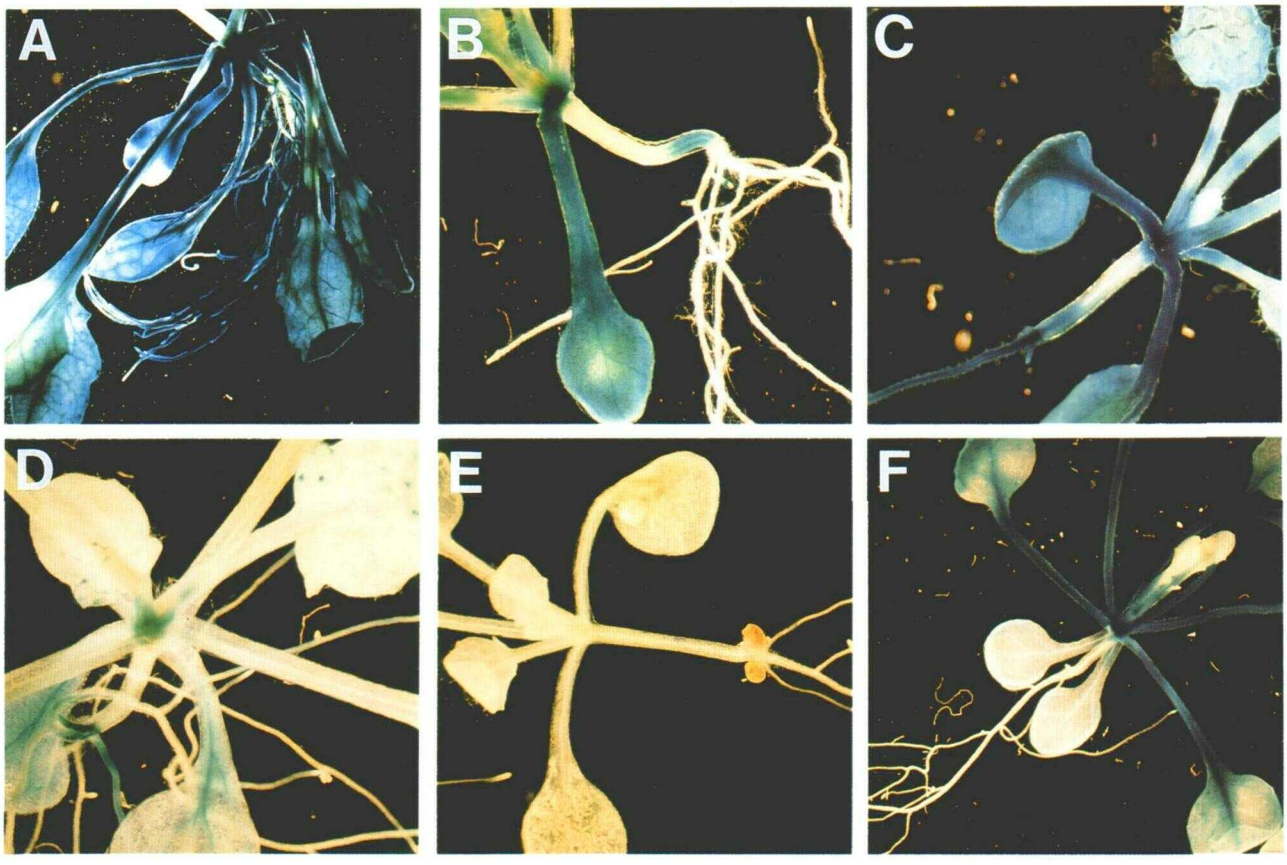

Figure 4. Histochemical Localization of GUS Activity in Transgenic Arabidopsis.

(A) and **(D)** Transgenic Arabidopsis plants containing the −861 *rd29A–GUS* fusion construct.
(B) and **(E)** Transgenic Arabidopsis plants containing the −946 *rd29B–GUS* fusion construct.
(C) and **(F)** Transgenic Arabidopsis plants containing the −268 *rd29A–GUS* and the 162-bp DNA fragment fused to the −61 *rd29A–GUS* fusion constructs, respectively.
Plants were grown on germination medium (GM) agar that contained 10 μg/mL kanamycin and were then exposed to dehydration for 8 hr (**[A]**, **[B]**, **[C]**, and **[F]**) or were grown under normal conditions (**[D]** and **[E]**). GUS activity is shown in rosette plants.

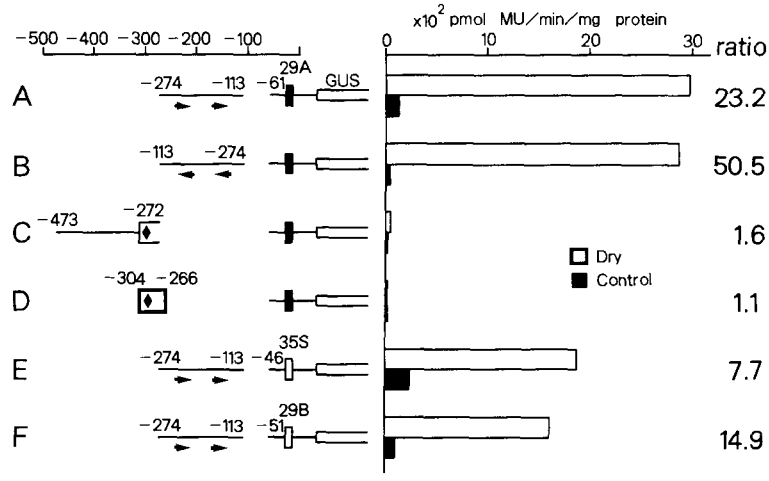

Figure 5. Analysis of the *rd29A* Promoter Region Involved in the Dehydration-Responsive Expression by a Gain-of-Function Experiment in Transgenic Tobacco.

DNA fragments of the rd29A promoter were fused to the −61 rd29A–GUS (from top to the 4th constructs), the −46 35S CaMV–GUS (the 5th construct), or the *−51 rd29B–GUS* (bottom construct) constructs and were introduced into tobacco chromosomes via Agrobacterium-mediated transformation. Leaves of transformed tobacco plants were dehydrated as described in Methods. Schematics of the chimeric constructs are shown on the left. Average GUS activities in tobacco leaves were measured before (filled squares) and after (open squares) the dehydration treatment; the results are shown at right. The multiplicities of induction of GUS by dehydration are shown on the right (ratio). GUS activity was measured in 15 independently obtained transgenic plants for each construct. Symbols are as given in the legend to Figure 2.

elements for dehydration-responsive expression. A 162-bp DNA fragment between −274 and −113 was fused to the −61 *rd29A–GUS* construct, and the fused construct was used for the transformation of tobacco and Arabidopsis plants. The −61 transformant showed no induction of GUS activity under conditions of water deficiency (Figure 2A). Transformants with the 162-bp DNA fragment fused to the −61 *rd29A–GUS* construct showed significant induction of GUS activity (23-fold) by dehydration stress (Figure 5). The 162-bp fragment worked in the reverse orientation at the same level (50-fold induction) as that in the forward orientation. Similar results were obtained with the −46 CaMV 35S promoter and with the −51 *rd29B* promoter, both of which contain minimum TATA box sequences (Figure 5). In contrast, the upstream 202-bp (−473 to −272) DNA fragment and the 39-bp (−304 to −266) conserved region were not involved in the induction of GUS activity under conditions of water deficiency (Figure 5).

The 162-bp DNA fragment contains a couple of 20-bp direct repeat sequences (Figure 3) but does not contain *cis*-acting elements that have been reported in plants. We then made an internal deletion series of the 162-bp DNA fragment to examine whether the 20-bp direct repeat sequence is essential for the induction of *rd29A* under conditions of water deficiency, as shown in Figure 6A. The 99-bp (−211 to −113) fragment, which contains one of the 20-bp direct repeat sequences, "downstream 20 bp," also functions in the expression of GUS activity (13.9-fold induction). Two 5′ deleted 99-bp fragments, the 70-bp (−182 to −113), and the 57-bp (−169 to −113) fragments, which contained the downstream 20 bp, functioned in the induction of *GUS* at multiplicities of 9.1 and 10.1, respectively. However, the 43-bp (−173 to −131) fragment that had lost both 20-bp direct repeat sequences did not respond to dehydration stress.

We then examined the 3′ deleted 162-bp DNA fragment for dehydration-responsive expression in transgenic tobacco. The 143-bp (−274 to −132) and 127-bp (−274 to −148) DNA fragments, which include two 20-bp direct repeats, both responded under conditions of water deficiency. The 84-bp (−274 to −191) fragment, which contains one of the 20-bp direct repeat sequences, "upstream 20 bp," still functioned in the dehydration-responsive expression of *GUS* (4.8-fold induction). However, the 56-bp (−274 to −219) DNA fragment with no 20-bp direct repeats did not function in the dehydration-responsive expression of *GUS*. These observations indicate that at least one of the 20-bp direct repeat sequences is necessary for the dehydration-responsive expression and that the downstream 20 bp seems to work more efficiently than the upstream 20 bp.

We observed low-level induction of GUS activity driven by promoter fragments containing either the downstream 20 bp or the upstream 20 bp (Figure 6B). When the 57-bp (−169 to −113) fragment containing the downstream 20 bp was tandemly duplicated, the level of GUS induction increased twofold over that obtained with single duplication. The 57-bp (−169 to −113) fragment that was duplicated three times exhibited an eightfold increase in GUS induction. However, the tandemly duplicated or triplicated 43-bp (−157 to −113) fragment without

the downstream 20 bp did not function in the induction of GUS activity (K. Yamaguchi-Shinozaki and K. Shinozaki, unpublished observation).

We then conducted a series of similar experiments with the 84-bp (−274 to −191) fragment that contains the upstream 20 bp. The tandemly triplicated 84-bp (−274 to −191) fragment functioned five times more efficiently than the duplicated fragment in the induction of GUS activity by dehydration (Figure 6B). Moreover, the level of this activity by dehydration increased from 4.8 to 23.3 with the triplicated 84-bp (−274 to −191) fragment. When the 67-bp (−274 to −219) DNA fragment with no 20-bp direct repeat was tandemly triplicated, the level of GUS activity did not respond to dehydration (K. Yamaguchi-Shinozaki and K. Shinozaki, unpublished observation). These observations indicate that either the downstream 20 bp or the upstream 20 bp functions as a dehydration-responsive regulatory, *cis*-acting element for the expression of *rd29A* and that the downstream 20 bp works more efficiently than the upstream 20 bp in induction.

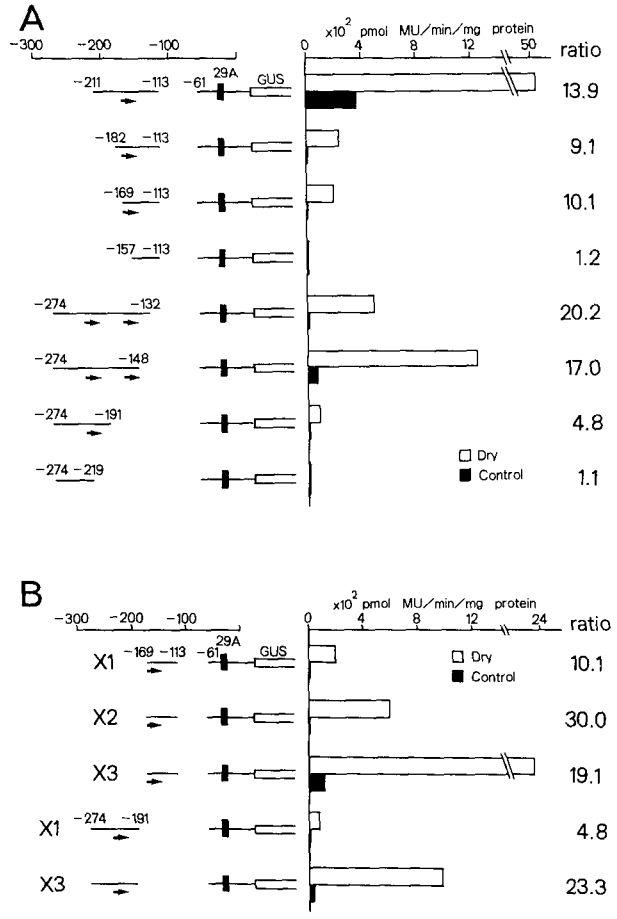

Figure 6. Role of the 20-bp Direct Repeat in Dehydration-Responsive Expression.

(A) Analysis of the role of the 20-bp direct repeat sequence in the dehydration-responsive expression of *rd29A* in transgenic tobacco. Tobacco leaves were transformed with DNA fragments containing the upstream 20 bp or the downstream 20 bp that had been fused to the −61 *rd29A–GUS* fusion construct. Experimental procedures are as described in the legend to Figure 5. GUS activity was measured in 15 independently obtained transgenic plants for each construct. Arrows indicate the 20-bp direct repeat sequences.

(B) Effects of copy number of the DNA fragments containing the 20-bp direct repeat in the dehydration-responsive expression. X1, X2, and X3 indicate chimeric constructs with the monomer, the tandemly repeated dimer, and the trimer of the 57-bp (−169 to −113) or the 84-bp (−274 to −191) fragment fused to the −61 *rd29A–GUS* construct, respectively. GUS activity was measured in 15 independently obtained transgenic plants for each construct. Arrows indicate the 20-bp direct repeat and rectangles indicate the TATA box.

Identification of a *cis*-Acting Element Involved in the Dehydration-Responsive Expression

We analyzed the mutated 20 bp in dehydration-responsive expression to test for a *cis*-acting element involved at the level of the nucleotide sequence. A 9-bp sequence (TACCGACAT) in the middle of the two 20-bp direct repeats was found to be identical, as shown in Figure 7A, and was designated as direct repeat 1 (DR1). We found DR1, with only one base change (A to T), in the promoter region of another dehydration-responsive gene, *rd17*, which is a member of a family of responsive to ABA (*rab*) or dehydrin genes (Shinozaki et al., 1993). We used the downstream 20 bp for further analysis, because the downstream 20 bp functions more efficiently than the upstream 20 bp. Because the 30-bp (−211 to −182) DNA fragment seems to contain an enhancer-like sequence (Figure 6A), we used the 71-bp (−215 to −145) DNA fragment to analyze the effect of the internal base substitution of the downstream 20 bp in the dehydration-responsive expression of the *GUS* reporter gene (Figure 7B). The base-substituted 71-bp fragments were fused to the −61 *rd29A–GUS* and were introduced into the tobacco chromosome by Agrobacterium-mediated transformation. We analyzed five mutated 71-bp fragments (M1 to M5) with four base substitutions around the downstream 20 bp. M1, M2, and M3 did not function in the dehydration-induced expression (1.5- to 1.9-fold increase), whereas M4 and M5 responded to dehydration stress (9.8- to 13.7-fold increase; Figure 7B). By contrast, the wild-type 71-bp sequence exhibited a 15.9-fold increase in the induction of GUS following dehydration. DR1 (TACCGACAT) in the downstream 20 bp was base-substituted in M1, M2, and M3 but not in M4 and M5. This indicates that DR1 functions as a regulatory element in the dehydration-responsive expression of *rd29A*.

We then examined whether DR1 can function alone as a positive *cis*-acting element for the dehydration-responsive expression of *rd29A*. We constructed a fusion gene with −61 *rd29A–GUS* using the 25-bp synthetic nucleotide (wild type) 5′-ACTACCGACATGAGTTCCAAAAAGC-3′ (−169 to −145), which was duplicated five times and which contains the downstream DR1 and its base-substituted mutant (MD2;

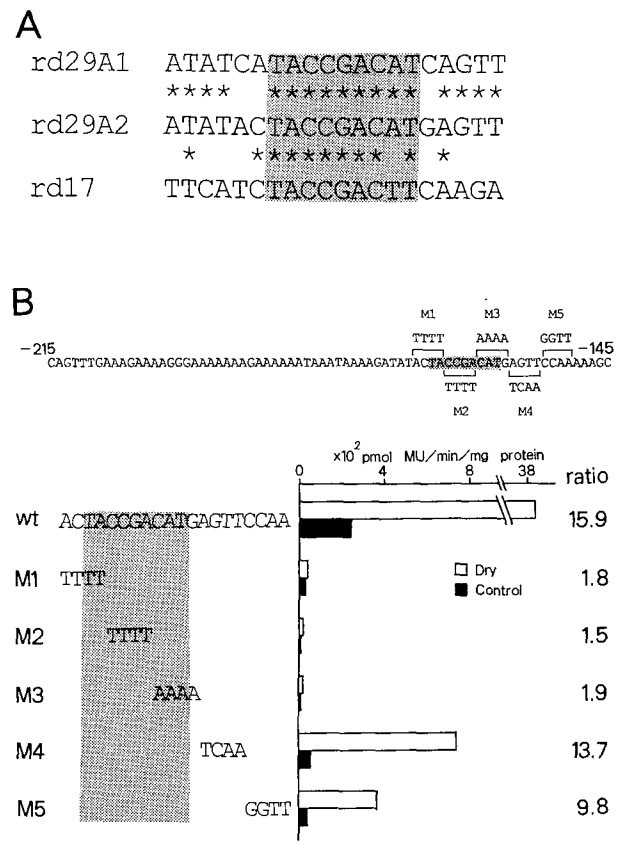

Figure 7. Identification of Dehydration-Responsive *cis* Element at the Nucleotide Sequence Level.

(A) Nucleotide sequences of the two conserved 9 bp (DR1) in the 20-bp direct repeats found in the *rd29A* promoter and its homologous sequence found in the *rd17* promoter that is responsive to dehydration. Asterisks indicate identical nucleotides, and the shaded box shows the 9-bp conserved sequence.

(B) Effects of base substitution in DR1 of the downstream 20 bp for the dehydration-responsive expression of *rd29A*. The tandemly repeated dimer of the 71-bp (−215 to −145) DNA fragments with the downstream 20 bp that contain base substitutions in DR1 (M1, M2, M3, M4, and M5) or do not contain the base substitutions (wt) were fused to the −61 *rd29A–GUS* construct and introduced into tobacco leaves. The experimental procedures are described in the legend to Figure 5. Shaded boxes and bold letters denote DR1 and its base-substituted sequences. GUS activity was measured in 15 independently obtained transgenic plants for each construct.

5′-ACTACAACCATGAGTTCCAAAAAGC-3′, bold letters indicating base substitutions). The fusion genes were then introduced into the tobacco chromosome. Transgenic tobacco plants containing the fusion genes were dehydrated for 5 hr and their GUS activities were measured before and after the dehydration treatment. The level of GUS activity driven by the wild-type 25 bp increased 7.8 times by dehydration treatment, while the mutant 25 bp did not function, as shown in Table 1. This result suggests that DR1 functions as a positive dehydration-responsive *cis*-acting element in the *rd29A* promoter and does not require other elements for its function.

DR1 Functions as a Dehydration-, High-Salt-, and Low-Temperature-Responsive Element but Not as an ABA-Responsive Element

We examined the effects of environmental stresses, such as low temperature, high salt, or the application of ABA as well as dehydration on the expression of the deleted *rd29A–GUS* fused gene in transgenic Arabidopsis. First, we analyzed the three deletion constructs, −861, −268, and −111 *rd29A–GUS*, and two internal deletion constructs, the 162-bp (−274 to −113) fragment in either direction fused to the −61 *rd29A–GUS*, concerning the dehydration-induced expression in transgenic Arabidopsis. RNA gel blotting was used to analyze the level of induction of the *GUS* gene driven by the deleted promoter of *rd29A* because it is superior to GUS activity measurements for determining the level of induction of the *GUS* mRNA. The GUS gene driven by the −861 and −268 *rd29A* promoters responded to low-temperature, high-salt, or ABA treatment as well as to dehydration, as shown in Figures 8A and 8B. The pattern of *GUS* expression induced by these stresses was similar to that of endogenous *rd29A*, and their maximum expressions were observed at 5 to 10 hr (data not shown). The −111 promoter did not respond to any of these conditions (Figure 8C). The 162-bp (−274 to −113) fragment fused to the −61 *rd29A–GUS*, which has minimum TATA box sequences, responded to low temperature, high salt, or dehydration but did not respond to exogenous ABA (Figures 8D and 8E). In this case, the maximum expression of *GUS* mRNA was observed at 1 to 2 hr, and the level of the mRNA decreased gradually (data not shown).

These results indicated that the 162-bp fragment contains a *cis*-acting element involved in low-temperature– or high-salt–responsive expression as well as the dehydration-responsive

Table 1. Dehydration-Responsive Expression of *rd29A–GUS* Constructs with DR1

rd29A–GUS Construct	Average GUS Activities[a] Untreated	Dehydrated	Ratio
Wild Type[b]	68.3	533.0	7.8
Mutant (MD2)[c]	60.0	61.5	1.0

The GUS reporter gene driven by the −61 *rd29A* promoter fused with the 25-bp fragment containing DR1 (wild type) or its mutated sequence (MD2) that is tandemly repeated five times in transgenic tobacco.

[a] Average GUS activities (picomole of product generated per minute per milligram of protein) were obtained from an analysis of 15 independently obtained tobacco plants for each construct.

[b] 5′-ACTACCGACATGAGTTCCAAAAAGC-3′.

[c] 5′-ACTACAACCATGAGTTCCAAAAAGC-3′.

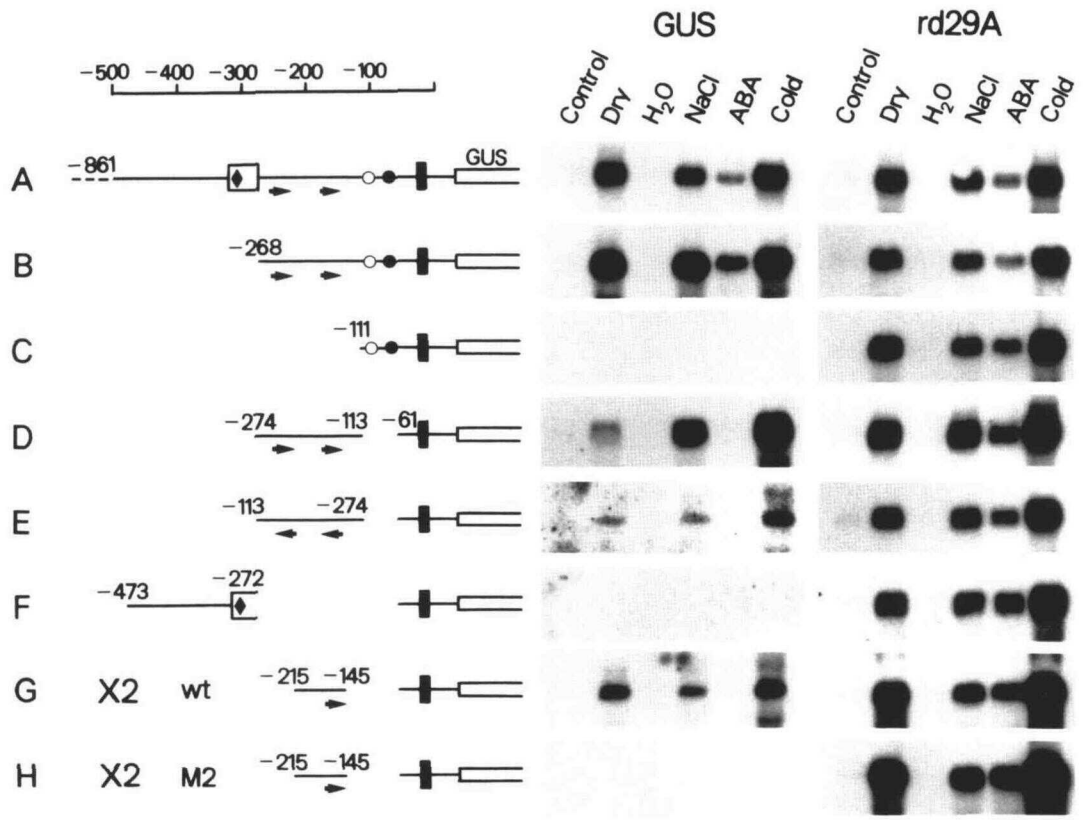

Figure 8. Analysis of the Effect of Various Treatments on the Induction of the *rd29A* Promoter–*GUS* Fusion Genes in Transgenic Arabidopsis.

Schematics of the chimeric constructs are shown on the left. RNA gel blotting was carried out to measure the amount of *GUS* mRNA or endogenous *rd29A* mRNA in transgenic Arabidopsis plants that had either been dehydrated (Dry) for 10 hr (A, B, and C) or 2 hr (D, E, F, G, and H); transferred from agar plates for hydroponic growth in water and treated for 10 hr (H_2O); transferred from agar plates for hydroponic growth in 250 mM NaCl (NaCl) and treated for 10 hr (A, B, and C) or 2 hr (D, E, F, G, and H); transferred from agar plates for hydroponic growth in 100 μM ABA for 10 hr (ABA); transferred to and grown at 4°C for 10 hr (Cold); or untreated (Control). RNA gel blotting was carried out as described in Methods. DNA fragments for the coding region of *GUS* or the 3′ flanking region of *rd29A* were used as probes. X2 indicates the chimeric construct with a tandemly repeated dimer of the 71-bp (−215 to −145) DNA fragment fused to the −61 *rd29A–GUS*. Symbols are as given in the legend to Figure 2.

expression but does not have an ABA-responsive *cis*-acting element. The upstream 202 bp (−473 to −272) had no effect in the expression of *GUS* (Figure 8F). The 71-bp (−215 to −145) fragment that contains the downstream DR1 (wild type) functioned in low-temperature– or high-salt–responsive expression and in dehydration-responsive expression but not in ABA-responsive expression, while its mutant 71 bp with mutated DR1 (M2; Figure 7B) did not function at all (Figures 8G and 8H). These results indicated that DR1 functions as a positive *cis*-acting element in low-temperature– or high-salt–responsive expression as well as in dehydration-responsive expression but not as an ABA-responsive element in the *rd29A* promoter.

Nuclear Protein Factors Involved in DNA Binding to DR1

We examined the nuclear protein factors that bind to the DR1 involved in the dehydration-induced expression of *rd29A*. Nuclear extracts were prepared from Arabidopsis rosette plants that had either been treated with a high-salt solution (0.25 M NaCl) for 5 hr or been left untreated. However, not enough nuclear fractions were obtained from the Arabidopsis plants dehydrated for 5 hr. We used the 162-bp (−274 to −113) DNA fragment as a probe that contains two 20-bp direct repeat sequences, which functions in dehydration-responsive expression (Figures 5 and 8). We detected a shifted band in the gel mobility

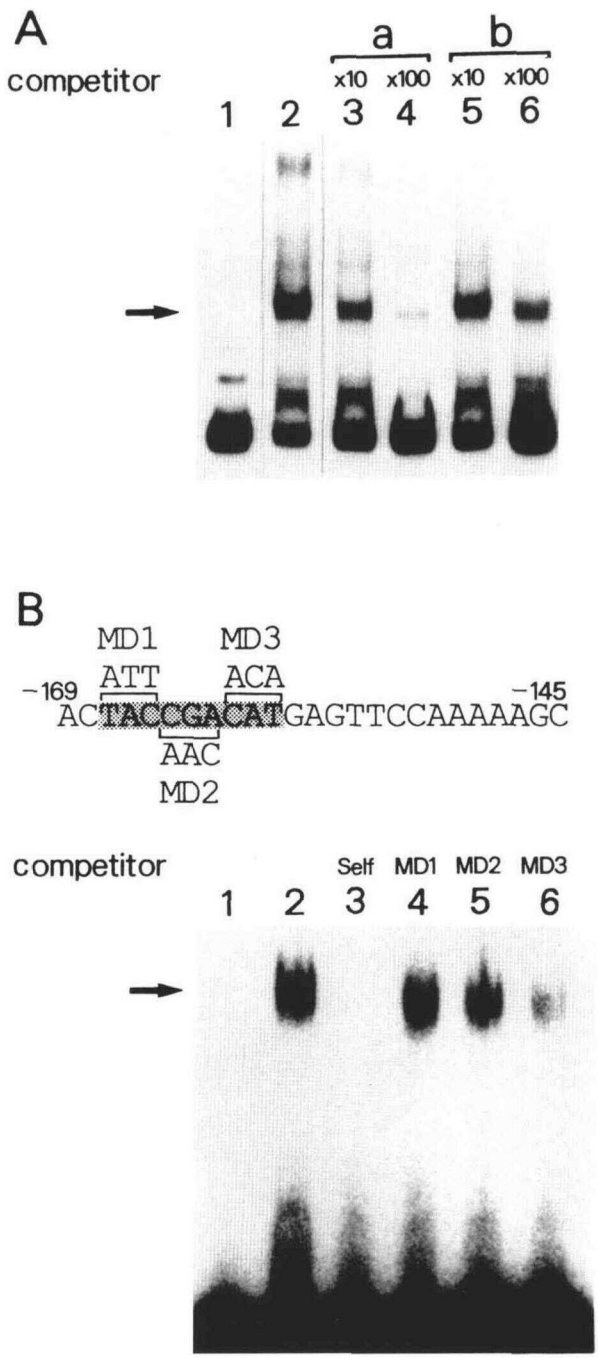

Figure 9. Identification of the DNA Binding Site of the Nuclear Factor.

(A) Identification of DNA binding proteins that bind specifically to the 162-bp (−274 to −113) region of the *rd29A* promoter. Nuclear extract was prepared from Arabidopsis rosette plants that had been transferred from agar plates for hydroponic growth in a 250-mM NaCl solution for 5 hr, as described in Methods. A gel retardation assay was carried out with the 162-bp DNA fragment as a probe, as described in Methods. The probe was incubated in the presence (lane 2) or absence (lane 1) of the nuclear extract. Cold competitors were also added as follows: lane 3, 10-fold amount of the 162-bp DNA fragment; lane 4, 100-fold amount of the 162-bp fragment; lane 5, 10-fold amount of the 202-bp (−473 to −272) DNA fragment ; lane 6, 100-fold amount of the 202-bp fragment. The arrow indicates the position of shifted band.

(B) Identification of the binding site of the nuclear factor in the downstream 20 bp. Two complementary synthetic oligonucleotides were annealed and used for the gel retardation assay. Nucleotide sequences of the probe (wild type) and its mutant sequences (MD1, MD2, and MD3) used as competitors are shown above. The gel retardation assay was performed as described in Methods. The radioactive wild-type probe was incubated in the presence (lane 2) or absence (lane 1) of the nuclear extract and electrophoresed in a 4% polyacrylamide gel. Nonradioactive competitors were added in the reaction mixtures as follows: lane 3, 100-fold amount of the probe DNA (self); lane 4, 100-fold of MD1; lane 5, 100-fold of MD2; lane 6, 100-fold of MD3. The arrow indicates the position of shifted band, and the shaded box denotes DR1.

shift assay using the 162-bp fragment as a probe and the nuclear extract prepared from Arabidopsis plants treated in the high-salt condition, as shown in Figure 9A. The same shifted band was detected using nuclear extract prepared from untreated Arabidopsis plants (K. Yamaguchi-Shinozaki and K. Shinozaki, unpublished data). The band was competed out with a 100-fold amount of the 162-bp DNA fragment itself but not with a 100-fold amount of the upstream 202-bp (−473 to −272) DNA fragment. This suggests that nuclear factors in either the salt-treated or untreated plants bind specifically to the 162-bp (−274 to −113) fragment, which contains the 20-bp direct repeat sequences involved in the dehydration-responsive expression of *rd29A*.

We then analyzed the binding site of the nuclear factor at the nucleotide sequence level in the 162-bp region. We used a 25-bp synthetic oligonucleotide, 5′-ACTACCGACATGAGTTC CAAAAAGC-3′ (−169 to −145; DR1 is underlined), that contains the downstream DR1 involved in the dehydration-responsive early expression of *rd29A*. We selected this 25-bp fragment on the basis of the deletion analysis of the *rd29A* promoter shown in Figure 7B and Table 1. A retardation band was obtained by incubation with nuclear extract prepared from salt-treated Arabidopsis plants (Figure 9B). The retardation band was competed out with a 100-fold amount of the same fragment but not with a 100-fold amount of the oligonucleotides with the base-substituted 25-bp sequences, MD1 (TAC to ATT) and MD2 (CGA to AAC). Weak competition was observed with MD3 (CAT to ACA). These results indicated that DR1 is important for the DNA binding of the nuclear factors.

DISCUSSION

A deletion analysis of the promoter regions of *rd29A* and *rd29B* in transgenic tobacco revealed that different *cis*-acting elements function in the dehydration-responsive expression of the two genes (Figures 2A and 2B). The 162-bp (−274 to −113) region is essential for the expression of *rd29A*, whereas the

169-bp region upstream of *rd29B* appears to function in dehydration-responsive expression. No conserved sequences were found in these regions (Figure 3). We analyzed one of the *cis*-acting elements responsible for the dehydration-induced expression of *rd29A* at the nucleotide sequence level. The deletion and the gain-of-function analysis of the promoter region of *rd29A* fused to the *GUS* reporter gene in transgenic tobacco and Arabidopsis revealed that the 20-bp direct repeat sequence in the 162-bp (−274 to −113) DNA fragment is necessary for dehydration-responsive expression (Figures 5 and 6). The base-substitution analysis revealed that the 9-bp conserved core sequence (DR1; TACCGACAT) in the 20-bp direct repeat sequence is essential for the regulation of the expression of *rd29A* under drought conditions (Figure 7; Table 1). Moreover, DR1 has been demonstrated to function as a *cis*-acting element involved in the induction of *rd29A* by either low-temperature or high-salt stress (Figure 8).

We found a DR1-related sequence in the promoter region of dehydration-responsive *rd17* that encodes a responsive to ABA (*rab*) or dehydrin homolog (Shinozaki et al., 1993). The level of induction by dehydration, high-salt, or low-temperature stress or by treatment with ABA was only slightly lower in *rd17* than in *rd29A*. By contrast, DR1 was not found in the promoter region of *rd29B*, which was not responsive to cold stress within 24 hr (Figure 1). Therefore, DR1 seems to be a *cis*-acting element involved in gene induction by dehydration, high salt, or low temperature. DR1 was designated as dehydration-, high-salt–, or low-temperature–responsive element (DRE) and functions as a positive *cis*-acting element that does not seem to require other elements for its function in the stress-inducible gene expression (Figure 8; Table 1). By contrast, *cis*-acting elements involved in signal-responsive expression, such as GT-1, hex1, chalcone synthase box II, and the G box, have been shown to require other *cis*-acting elements for their function (Block et al., 1990; Lam and Chua, 1990, 1991; Oeda et al., 1991). DR1 does not function as an ABA-responsive element in the induction of *rd29A* (Figure 8). Under the stress conditions tested, ABA does not seem to function in the DR1-mediated process in the induction of *rd29A*. RNA gel blot analysis has indicated that the rapid response of *rd29A* to dehydration is ABA independent and that its slow induction is ABA responsive (Yamaguchi-Shinozaki and Shinozaki, 1993a). The 71-bp (−215 to −145) fragment fused to the −61 *rd29A–GUS* responded rapidly to dehydration as well as to high salt (K. Yamaguchi-Shinozaki and K. Shinozaki, unpublished data), indicating that DRE is a *cis*-acting element involved in the rapid ABA-independent response of *rd29A* to dehydration.

Nuclear factors that specifically bind to DR1 have been identified by gel shift assay with nuclear extract prepared from Arabidopsis plants treated in high-salt conditions (Figure 9) and have been designated as DRE Binding Factor 1 (DRBF1). The DNA binding factor was also detected in the nuclear fraction prepared from unstressed, normal Arabidopsis plants. The DNA binding activity of the nuclear factor of salt-treated plants was similar to that of untreated plants. These observations suggest that DRBF1 are constantly present in the nucleus and bind to DR1 but only act to stimulate the transcription of *rd29A* under drought conditions. There are several possible roles for the DRBF1 that interacts with DR1 in the dehydration-responsive expression of *rd29A*: DRBF1 interacts with either a positive factor in the stress condition or with a negative factor in the normal growth condition. DRBF1 can undergo reversible modification under conditions of dehydration, low temperature, or high salt. We are now in the process of cloning cDNAs that encode DRBF1, using protein gel blots probed with DNA to identify more precisely the role of DRBF1 in the stress-responsive expression of *rd29A*.

RNA gel blot analysis indicated that the second slow induction of *rd29A* is dependent on ABA (Yamaguchi-Shinozaki and Shinozaki, 1993a). The 162-bp (−274 to −113) fragment fused to the −61 *rd29A–GUS* did not respond to exogenous ABA, while the −268 *rd29A–GUS* was induced by ABA (Figure 8). These observations suggest that the 53-bp (−113 to −61) region contains *cis*-acting elements involved in the ABA-responsive, slow induction of *rd29A*. In this region, we found one ABRE and one as1. A conserved sequence, YACGTGGC, has been reported to function as an ABRE in many ABA-responsive genes (Marcotte et al., 1989; Mundy et al., 1990; Bray, 1991). However, the −111 *rd29A–GUS* was not induced by dehydration or ABA, which suggests that the −111 promoter region does not contain all the *cis* elements involved in ABA-responsive expression and requires other *cis* elements in the 158-bp (−268 to −111) region for its function.

The *rd29B* gene responds to dehydration stress slowly and is probably induced by endogenous ABA produced under drought conditions (Yamaguchi-Shinozaki and Shinozaki, 1993a; Figure 1), while the −169 *rd29B–GUS* is slowly induced by dehydration or exogenous ABA treatment (K. Yamaguchi-Shinozaki and K. Shinozaki, unpublished data). We found two ABREs and one myb recognition sequence in the −169 promoter region of *rd29B*. Recently, we isolated a gene encoding a myb homolog (*Atmyb2*) that is induced by drought and is probably involved in the regulation of dehydration-responsive genes in Arabidopsis (Urao et al., 1993). Protein synthesis is necessary for the ABA-dependent expression of *rd29B* (K. Yamaguchi-Shinozaki and K. Shinozaki, unpublished data), which suggests that the myb recognition sequence as well as ABRE may function in ABA-dependent induction of *rd29B*.

Figure 10 shows a schematic model of the signal transduction pathways between the expression of *rd29A* and *rd29B* and the initial signal of environmental stresses, such as dehydration, low temperature, or high salt. The *rd29A* promoter contains at least two *cis*-acting elements that are involved in the induction of *rd29A* by dehydration, high salt, or low temperature. One of these elements is DR1 (TACCGACAT), which functions in the first rapid response of *rd29A* to the environmental signal. ABA is not involved in this process. The other element is located in the 53-bp (−113 to −61) region containing one ABRE and one as1. These elements probably function in the second slow induction of *rd29A*. ABA appears to mediate this slow response of *rd29A* against environmental stresses. The *rd29B* gene exhibited only the slow response to dehydration

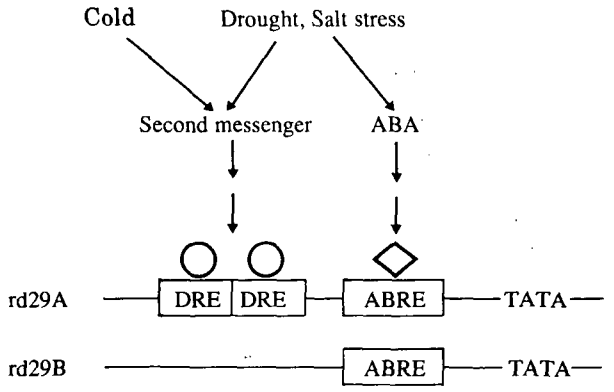

Figure 10. Schematic Representation of the Induction of Two *rd29* Genes and Their *cis*-Acting Elements Involved in Stress-Responsive Expression.

There are at least two independent signal transduction pathways, which are ABA independent and ABA responsive, between the environmental stresses and the expression of the two *rd29* genes. Different *cis-acting elements, DRE and ABRE, may function in the* ABA-independent and ABA-responsive induction of *rd29A*, whereas ABRE seems to be involved in the ABA-responsive expression of *rd29B*.

or salt stress at 24 hr but did not respond significantly to low temperature within 24 hr. ABA may be involved in part in the slow expression of *rd29B*. The −169 promoter region of *rd29B* is necessary for its expression and contains two ABREs and one myb recognition sequence. This region corresponds to the 53-bp region of *rd29A* and has several conserved sequences (Figure 3). This model indicates that different *cis*-acting elements are responsible for the different expression patterns of *rd29A* and *rd29B* under conditions of dehydration, high salt, or low temperature.

METHODS

Plant Growth and Stress Treatment

Arabidopsis thaliana (Columbia ecotype) was grown on germination medium (GM) agar plates (Valvekens et al., 1988) at 22°C for 3 weeks and was used in stress treatment experiments prior to bolting. Arabidopsis rosette plants were harvested from GM agar plates and were then dehydrated on Whatman 3MM paper at 22°C and 60% humidity under dim light. Plants subjected to treatment with abscisic acid (ABA) and to salt stress were grown hydroponically in solution containing 100 μM ABA and 250 mM NaCl, respectively, under dim light. Cold treatment was conducted under dim light by exposure of plants grown at 22°C to a temperature of 4°C. In each case, the plants were subjected to the stress treatments for various periods and were frozen in liquid nitrogen.

Isolation of RNA from Arabidopsis and RNA Gel Blot Analysis

Total RNA was isolated according to the method of Nagy et al. (1988). RNA gel blot hybridizations were performed as described previously by Yamaguchi-Shinozaki et al. (1989).

Construction of Deleted or Base-Substituted Promoter Regions of Two *rd29* Genes Fused to a β-Glucuronidase Reporter Gene

The construction of a chimeric gene with the *rd29A* promoter 5′ deleted to −861 fused to β-glucuronidase (*GUS*) (the −861 *rd29A-GUS*) has been described previously by Yamaguchi-Shinozaki and Shinozaki (1993a). The fusion gene contains an 861-bp region upstream from the site of initiation of transcription, 80 bp of the untranslated leader sequence, and 17 bp of the coding region of *rd29A*. Reexamination by primer extension has revealed that the correct initiation site is 19 bp upstream from that described by Yamaguchi-Shinozaki and Shinozaki (1993b). The −946 *rd29B-GUS* fusion gene was constructed by the ligation of a 1045-bp HincII fragment, which contained the 946 bp upstream from the site of initiation of transcription, 85 bp of the untranslated leader sequence, and 14 bp of the coding region of *rd29B*, into the SmaI site of pBI101. Deletion of the 5′ end of the *rd29A* and *rd29B* promoter fragments in pBI101 were obtained by exonuclease III and mung bean nuclease digestion at the BamHI site of pBI101. To protect the upstream sequence from nuclease digestion, the SalI site of pBI101 was modified with a thio derivative.

A 162-bp AflIII-AvaII fragment between −274 and −113 from the transcriptional initiation site of *rd29A* was fused to the −61 *rd29A-GUS* construct (Figure 2A), the −46 cauliflower mosaic virus (CaMV) 35S-*GUS* construct (Fang et al., 1989), or the −51 *rd29B-GUS* construct (Figure 2B) using a HindIII linker. A 202-bp SspI-AvaII fragment between −473 and −272 was fused to the −61 *rd29A-GUS* construct using a HindIII linker. A 39-bp (−304 to −266) fragment derived from the *rd29A* promoter was prepared by annealing complementary oligonucleotides with HindIII linkers at both ends and ligated to the HindIII site of the −61 *rd29A-GUS* construct. Deleted fragments of the 162-bp (−274 to −113) AflIII-AvaII fragment were obtained by digestion with exonuclease III and mung bean nuclease and fused to the −61 *rd29A-GUS* construct using a HindIII linker.

A 71-bp (−215 to −145) fragment of *rd29A* (wild type) and its mutant fragments (M1 to M5) were prepared by polymerase chain reaction and were fused to the −61 *rd29A-GUS* construct. The primers used for the amplification of the 71-bp wild-type fragment were 5′-AAGCTTACATCAGTTTGAAAGAAA and AAGCTTGCTTTTTGGAACTCATG-TC-3′. Primers containing mutation used for the amplification of the 71-bp mutant fragments were 5′-AAGCTTGCTTTTTGGAACTCATGTCGGAAAAATATCTTTTATTTATTTTTTC-3′ (M1), 5′-AAGCTTGCTTTTTGGAACTCATGAAAATAGTATATCTTTTATTTATTTTTTC-3′ (M2), 5′-AAGCTTGCTTTTTGGAACTTTTTTCGGTAGTATATCTTTTATTTA-3′ (M3), 5′-AAGCTTGCTTTTTGGTTGACATGTCGGTAGTATATCTTTTA-3′ (M4), and 5′-AAGCTTGCTTTAACCAACTCATGTCGGTAGTATATCT-3′ (M5; see Figure 7B). A 25-bp (−167 to −143) fragment derived from the *rd29A* promoter and its mutant (MD2) were prepared by annealing complementary oligonucleotides with HindIII linkers at both ends and were ligated to the HindIII site of the −61 *rd29A/GUS* construct. The structures of the fusion constructs were confirmed by sequencing the boundary sites of the fused gene from both the multicloning site in pBI101 (primer:

5′-CTCGTATGTTGTGTGGAATTGT-3′) and a sequence from the *GUS* coding region (primer: 5′-TCACGGGTTGGGGTTTCTAC-3′) as primers.

Transgenic Plants

pBI101 plasmids containing the promoter–*GUS* fusion constructs were transferred from *Escherichia coli* DH5α into *Agrobacterium tumefaciens* via triparental mating with an *E. coli* strain that contained the mobilization plasmid pRK2013. The pBI101 vectors containing the promoter–*GUS* fusion construct was transferred into Agrobacterium strain LBA4404 for transformation of tobacco and into Agrobacterium strain C58 for transformation of Arabidopsis. Transformation of *Nicotiana tabacum* cv SR1 and Arabidopsis (Colombia ecotype) was performed as described previously (Valvekens et al., 1988; Benfey et al., 1989). Primary transgenic explants of Arabidopsis and tobacco were grown at 22 and at 25°C, respectively, under a 16-hr light/8-hr dark cycle. The transgenic plants were analyzed for integration of the intact promoter–*GUS* chimeric gene into the genomic DNA by polymerase chain reaction with a boundary sequence from the multicloning site in pBI101 (5′-CTCGTATGTTGTGTGGAATTGT-3′) and a sequence from the *GUS* coding region (5′-TCACGGGTTGGGGTTTCTAC-3′) as primers and genomic DNA as a template (data not shown).

Assays of GUS Activity

GUS activity was assayed in tissue extracts by fluorometric quantitation of 4-methylumbelliferone produced from the glucuronide precursor using a standard protocol (Jefferson et al., 1986). GUS activity was expressed in picomoles of product generated per minute per milligram of protein. Histochemical localization of GUS activity in situ was performed by incubating samples of plant tissue in 5-bromo-4-chloro-3-indolyl glucuronide at 37°C, fixing the samples in 0.3% formaldehyde, and removing the chlorophyll from green tissues by incubation in 50 to 100% ethanol.

RNA Gel Blot Analysis of the Accumulation of the *GUS* mRNA in Transgenic Arabidopsis

Transgenic Arabidopsis rosette plants grown in GM agar plates were subjected to various environmental stresses, such as dehydration, low temperature, high salt, or exogenous ABA, for the indicated times and were frozen in liquid nitrogen. RNA was extracted from the frozen plants as described previously by Yamaguchi-Shinozaki et al. (1990). RNA gel blot hybridization was performed, as described above, using the DNA fragment containing the coding region of the *GUS* reporter gene or a fragment containing the 3′ flanking region of *rd29A* as probes.

Gel Retardation Assay of DNA Binding Proteins in Arabidopsis Nuclear Extract

Nuclear protein extracts were prepared from Arabidopsis rosette plants treated with 250 mM NaCl solution for 5 hr, as described elsewhere by Green et al. (1989). The DNA binding reaction was performed as described previously by Green et al. (1987). The reaction mixture was subjected to electrophoresis on a 4% polyacrylamide gel in 0.25 × Tris-borate EDTA buffer at 100 V for 2 hr. The gel was dried and subjected to autoradiography. The probes and competitors used for the experiments are described in the legend to Figure 9.

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